

**[0009]** On the other hand, a skin lotion for anti-aging consisting of a polymerization product with an average molecular weight of 280 to 20000 of a tripeptide composed of one glycine residue and two other amino acid residues in a degradation product of collagen or gelatin obtained by collagenase (see Japanese Patent Laid-Open No. 2000-309521), a collagen-producing promoter comprising a mixture of tripeptides consisting of (Gly-Ala-Arg), (Gly-Ala-Hyp), (Gly-Ala-Lys), (Gly-Pro-Ala), (Gly-Pro-Arg), (Gly-Pro-Hyp), and (Gly-Pro-Ser) as an active ingredient (see Japanese Patent Laid-Open No. 2003-137807) and elsewhere are known as physiologically active substances comprising an oligopeptide or a polymerization product thereof as an active ingredient. However, no epithelial cell growth promoter comprising a tripeptide as an active ingredient has been proposed so far.

#### DISCLOSURE OF THE INVENTION

**[0010]** An object of the present invention is to provide a novel water-soluble oligopeptide that can be produced in a relatively simple way, has not only the hair growth-promoting effect but also the epithelial cell growth-promoting effect such as skin regeneration, and can easily pass through the stratum corneum to reach and act on a desired target cell.

**[0011]** The inventors have conducted intensive studies to develop chemicals useful as a hair growth agent and, as a result, have arrived at a discovery that an active substance exhibiting excellent hair growth effect is present in an extract from the culture supernatant of bacteria of the genus *Bacillus*, that the hair growth effect results from a particular oligopeptide structure in this active substance, and that any particular polypeptide having this oligopeptide structure not only exhibits desired hair growth effect but also exhibits the effect of promoting cell regeneration in skin transplantation or in the restoration of skin ulcer or aging skin. The inventors have further arrived at a discovery that a water-soluble oligopeptide having particular amino acid units exhibits excellent epithelial cell growth-promoting effect leading to completion of the present invention on the basis of these discoveries.

**[0012]** Namely, the present invention provides a water-soluble oligopeptide and a water-soluble salt thereof having 3-7 amino acid units containing an isoleucyl-glycyl-seryl unit, 4-7 amino acid units containing a prolyl-

isoleucyl-glycyl unit and a seryl unit or 5-7 amino acid units containing a glycyl-prolyl-isoleucyl-glycyl unit and a seryl unit or a threonyl unit. Further the present invention provides an epithelial cell growth promoter comprising at least one selected from the water-soluble oligopeptide and the water-soluble salt thereof as an active ingredient.

**[0013]** The effect of the water-soluble oligopeptide in the epithelial cell growth promoter of the present invention is exerted not only in the oligopeptide *per se* but also in a polypeptide having these oligopeptide units as its molecular structure unit. However, those having a molecular weight of 500 or more are rendered slightly soluble in water so that they are not preferable as a hair growth agent.

**[0014]** Among such water-soluble oligopeptides, a tripeptide includes isoleucyl-glycyl-serine and prolyl-isoleucyl-glycine. And, a tetrapeptide is exemplified, for example, by this tripeptide with an amino acid residue such as a glycyl, alanyl, arginyl, asparagyl, lysyl, seryl, valyl, or glutamyl group bonded to the front or rear, of which glycyl-prolyl-isoleucyl-glycine (SEQ ID NO: 1) and prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 2) are preferred.

**[0015]** A pentapeptide is exemplified, for example, by the glycyl-prolyl-isoleucyl-glycyl (SEQ ID NO: 1) group with an amino acid residue such as a seryl group or a threonyl group bonded to the front or rear, of which glycyl-prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 3) and glycyl-prolyl-isoleucyl-glycyl-threonine (SEQ ID NO: 4) are preferred.

**[0016]** Further, preferable hexapeptide and heptapeptide are any of those having the above-mentioned pentapeptide unit at the carboxyl terminus, such as, for example, alanyl-glycyl-prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 5), seryl-glycyl-prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 6), glycyl-seryl-glycyl-prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 7) and the like.

**[0017]** The water-soluble oligopeptide of the present invention may be in a free form or a water-soluble salt form. This water-soluble salt includes, for example, sodium salt, potassium salt, lithium salt, ammonium salt and the like.

**[0018]** The oligopeptide of the present invention can be produced by reacting a raw material amino acid having the  $\alpha$ -amino group protected and an amino acid having the carboxyl group protected by any conventional method for forming a peptide bond in synthesis of a polypeptide such as, for example, a condensation method, active ester method, azide method,

mixed acid anhydride method and others, so as to form a peptide, which is in turn repeatedly subjected to the step of eliminating the protecting groups.

**[0038]** Figure 2 is a mass spectrum of the tripeptide obtained in Example 1.

**[0039]** Figure 3 is a mass spectrum of the tripeptide obtained in Reference Example 1.

**[0040]** Figure 4 is a mass spectrum of the tetrapeptide obtained in Example 2.

**[0041]** Figure 5 is a mass spectrum of the tetrapeptide obtained in Reference Example 2.

**[0042]** Figure 6 is a mass spectrum of the pentapeptide obtained in Example 3.

**[0043]** Figure 7 is a bar graph showing the effect of the IGS obtained in Example 5 on HBKs growth.

**[0044]** Figure 8 is a bar graph showing the effect of the PIG obtained in Example 5 on HBKs growth.

**[0045]** Figure 9 is a bar graph showing the effect of the PIGS obtained in Example 5 on HBKs growth.

**[0046]** Figure 10 is a bar graph showing the effect of the GPIG obtained in Example 5 on HBKs growth.

**[0047]** Figure 11 includes computer-processed diagrams of photographs taken with a digital camera and showing the shaved areas with the group (B) coated with application of the epithelial cell growth promoter obtained in Example 6 and the control group (A) on the 14th day from which the ratio of the hair-regrowth area was calculated as percentages using an image analysis software.

**[0048]** Figure 12 is a bar graph showing the effect of cell growth on the HBKs obtained in Example 7.

**[0049]** Figure 13 is a bar graph showing the effect of cell growth on the epidermal cells obtained in Example 7.

**[0050]** Figure 14 is a bar graph showing the effect of cell growth on the dermal fibroblasts obtained in Example 7.

**[0051]** Figure 15 is a bar graph showing the effect of cell growth on the hair papilla cells obtained in Example 7.

#### BEST MODE FOR CARRYING OUT THE INVENTION

**[0052]** In the following, the best mode for carrying out the present invention will be described by way of Examples. However, the present invention is not intended to be limited by them in any way.

### Example 1

**[0053]** Tetrahydrofuran and dicyclohexylcarbodiimide were used as a solvent and a condensing agent, respectively, to produce benzyl ester of N-Fmoc-isoleucyl-glycyl-serine by successively reacting benzyl ester of serine having the hydroxyl group protected with a *tert*-butyl group, glycine having the  $\alpha$ -amino group protected with a 9-fluorenylmethoxycarbonyl group (hereinafter, abbreviated to Fmoc group), and isoleucine having the  $\alpha$ -amino group protected with an Fmoc group in the presence of triethylamine by use of an automatic solid-phase synthesizer (Model "Syro 2000", manufactured by MultiSynTech Co.).

**[0054]** After the termination of reaction, the elimination of the protecting groups and the hydrolysis of the ester were performed by treating the generated intermediate with hydrofluoric acid in a mixture solvent of methyl alcohol and dioxane (3:1 by volume) and purifying it by chromatography, to obtain racemic isoleucyl-glycyl-serine. In this case, the yield thereof was approximately 46%.

**[0055]** Next, this tripeptide was passed through a C<sub>18</sub> column [Model "HP1100" (3.0×250 mm), manufactured by Hewlett-Packard Co.] (the column was used for purity analysis in Examples 2 to 4 below), and components adsorbed thereon were eluted at a flow rate of 0.4 ml/minute for 20 minutes with a solution of acetonitrile in a concentration ranging from 0 to 30% containing 0.1% trifluoroacetic acid. As a result, the tripeptide was eluted with a retention time of 11.671 minutes in a purity of 95.87%.

**[0056]** The mass of this tripeptide was analyzed with a MALDI-MS mass spectrometer [Model "Dynamo", manufactured by Thermo BioAnalysis Corp.] (the mass spectrometer was used for mass spectrometry in Examples 2 to 4 below) to show that the mass ( $m/z$ ,  $MH^+$ ) was  $m/z$  275.339. The result of this mass spectrometry is shown in Figure 2.

### Reference Example 1

**[0057]** Benzyl ester of glycine, isoleucine having the  $\alpha$ -amino group protected with an Fmoc group, and proline having the  $\alpha$ -amino group protected with an Fmoc group were used to produce racemic prolyl-isoleucyl-glycine by totally the same procedure as in Example 1. In this case, the yield thereof was approximately 40%.

**[0058]** Next, this tripeptide was passed through a C<sub>18</sub> column, and components adsorbed thereon were eluted at a flow rate of 0.4 ml/minute for 20 minutes with a solution of acetonitrile in a concentration ranging from 0 to 30% containing 0.1% trifluoroacetic acid. As a result, the tripeptide was eluted with a retention time of 14.052 minutes in a purity of 95.93%.

**[0059]** The mass of this tripeptide was analyzed to show that the mass ( $m/z$ ,  $MH^+$ ) was  $m/z$  285.102. The result of this mass spectrometry is shown in Figure 3.

#### Example 2

**[0060]** Benzyl ester of serine having the hydroxyl group protected with a *tert*-butyl group, glycine having the  $\alpha$ -amino group protected with an Fmoc group, isoleucine having the  $\alpha$ -amino group protected with an Fmoc group, and proline having the  $\alpha$ -amino group protected with an Fmoc group were used to produce racemic prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 2) by totally the same procedure as in Example 1. In this case, the yield thereof was approximately 54%.

**[0061]** Next, this tetrapeptide was passed through a C<sub>18</sub> column, and components adsorbed thereon were eluted at a flow rate of 0.4 ml/minute for 20 minutes with a solution of acetonitrile in a concentration ranging from 0 to 40% containing 0.1% trifluoroacetic acid. As a result, the tetrapeptide was eluted with a retention time of 12.313 minutes in a purity of 95.11%.

**[0062]** The mass of this tetrapeptide was analyzed to show that the mass ( $m/z$ ,  $MH^+$ ) was  $m/z$  373.963. The result of this mass spectrometry is shown in Figure 4.

#### Reference Example 2

**[0063]** Benzyl ester of glycine, isoleucine having the  $\alpha$ -amino group protected with an Fmoc group, proline having the  $\alpha$ -amino group protected with an Fmoc group, and glycine having the  $\alpha$ -amino group protected with an Fmoc group were used to produce racemic glycyl-prolyl-isoleucyl-glycine (SEQ ID NO: 1) by totally the same procedure as in Example 1. In this case, the yield thereof was approximately 48%.

**[0064]** Next, this tetrapeptide was passed through a C<sub>18</sub> column, and components adsorbed thereon were eluted at a flow rate of 0.4 ml/minute for 20 minutes with a solution of acetonitrile in a concentration ranging from

5 to 40% containing 0.1% trifluoroacetic acid. As a result, the tetrapeptide was eluted with a retention time of 11.648 minutes in a purity of 99.60%.

**[0065]** The mass of this tetrapeptide was analyzed to show that the mass ( $m/z$ ,  $MH^+$ ) was  $m/z$  343.986. The result of this mass spectrometry is shown in Figure 5.

### Example 3

**[0066]** Tetrahydrofuran and dicyclohexylcarbodiimide were used as a solvent and a condensing agent, respectively, to produce benzyl ester of N-Fmoc-glycyl-prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 3) by successively reacting benzyl ester of serine having the hydroxyl group protected with a *tert*-butyl group, glycine having the  $\alpha$ -amino group protected with an Fmoc group, isoleucine having the  $\alpha$ -amino group protected with an Fmoc group, proline having the  $\alpha$ -amino group protected with an Fmoc group, and glycine having the  $\alpha$ -amino group protected with an Fmoc group in the presence of triethylamine by use of an automatic solid-phase synthesizer (Model "Syro 2000", manufactured by MultiSynTech Co.).

**[0067]** After the termination of reaction, the elimination of the protecting groups and the hydrolysis of the ester were performed by treating the generated intermediate with hydrofluoric acid in a mixture solvent of methyl alcohol and dioxane (3:1 by volume) and purifying it by chromatography, to give racemic glycyl-prolyl-isoleucyl-glycyl-serine. In this case, the yield thereof was approximately 40%.

**[0068]** Next, this pentapeptide was passed through a  $C_{18}$  column [Model "Discovery  $C_{18}$ " (4.6×250 mm), manufactured by Supelco], and components adsorbed thereon were eluted at a flow rate of 1.5 ml/minute for 20 minutes with a solution of acetonitrile in a concentration ranging from 2 to 22% containing 0.1% trifluoroacetic acid. As a result, the pentapeptide was eluted with a retention time of 9.761 minutes in a purity of 96.9%.

**[0069]** The mass of this pentapeptide was analyzed with an LC-MS mass spectrometer [LC portion: Model "Agilent 1100 Series", manufactured by Agilent Technologies, Ltd.; MS portion: Model "ThermoFinnigan LCQ advantage (software Xcalibur)", manufactured by Thermo Electron Corp.] by cation measurement using an electrospray ionization method to show

that the mass ( $m/z$ ,  $MH^+$ ) was  $m/z$  430.1. The result of this mass spectrometry is shown in Figure 6.

#### Example 4

**[0070]** Racemic glycyl-prolyl-isoleucyl-glycyl-threonine (SEQ ID NO: 4) was produced by totally the same procedure as in Example 1 except that benzyl ester of threonine having the hydroxyl group protected with a *tert*-butyl group was used instead of the benzyl ester of serine having the hydroxyl group protected with a *tert*-butyl group. The mass ( $m/z$ ,  $MH^+$ ) of this pentapeptide was  $m/z$  444.3.

#### Example 5

**[0071]** The epithelial cell growth promoters of Examples 1 and 2 and Reference Examples 1 and 2 were subjected to a cell growth test for murine HBKs, respectively. The following culture medium and test medium were used.

##### (a) Culture medium

DMEM (product code "D-5523", a product by Sigma)

FBS (product code "10100756", a product by Cansera International Inc.)  
10%

Penicillin/streptomycin (product code "15140-122", a product by GIBCO)  
1%

##### (b) Test medium

MCDB153 (product code "M7403", a product by Sigma)

Insulin (product code "16634", bovine, a product by Sigma) 5  $\mu$ g/ml

Apo-transferrin (product code "T1147", human, a product by Sigma)  
10  $\mu$ g/ml

EGF (product code "01-101", murine, a product by Upstate Biotechnology Inc.)  
5 ng/ml

BPE (product code "13028-014", bovine pituitary extract, a product by GIBCO)  
35  $\mu$ g/ml

Water-soluble hydrocortisone (product code "174-00", a product by NACALAI TESQUE INC.)  
0.5  $\mu$ g/ml



## (2) Cell growth test

### (i) Sample preparation

**[0075]** The isoleucyl-glycyl-serine (hereinafter, abbreviated to IGS), prolyl-isoleucyl-glycine (hereinafter, abbreviated to PIG), prolyl-isoleucyl-glycyl-serine (SEQ ID NO: 2) (hereinafter, abbreviated to PIGS), and glycyl-prolyl-isoleucyl-glycine (SEQ ID NO: 1) (hereinafter, abbreviated to GPIG) obtained in Examples 1 and 2 and Reference Examples 1 and 2 were separately dissolved in a test media to prepare 6 types of sample solutions each in a concentration of 0.3  $\mu$ M, 1  $\mu$ M, 3  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, and 100  $\mu$ M. Only a test medium was used as a control.

### (ii) HBK growth test

**[0076]** After 24 hours from the inoculation of the cells, the culture solutions were removed, and the cultured cells were washed with an MCDB153 solution. Each of the above-mentioned sample solutions was added to the cultured cell at 100  $\mu$ l/well and cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**[0077]** After 4 days, AlamarBlue reagent (registered trademark, cat. No. DAL1100, lot No. AB083002, a product by Biosource International Inc.) was added at 10  $\mu$ l/well, and the culture was continued at 37°C in a 5% CO<sub>2</sub> atmosphere. After culturing for 2 hours, fluorescence intensity (excitation wavelength: 544 nm, measurement wavelength: 590 nm) was measured with a microplate reader (Model "Fluoroskan Ascent FL", manufactured by Labsystems) to evaluate the number of cells.

**[0078]** Incidentally, the evaluation was performed by calculating the ratios (percentage) of the degrees of cell growth for the peptides obtained in Examples 1 and 2 and Reference Examples 1 and 2 relative to the degree of cell growth for the control and indicating the cell growth ratios by mean value $\pm$ standard deviation (n=5). A significant difference test was performed by Dunnett's multiple comparison test (software "Super ANOVA V. 1.11", a product by Abacus Concepts). When a significance level was less than 5% (p<0.05), it was concluded that there was significant difference. The results with the IGS, the results with the PIG, the results with the PIGS and the results with the GPIG obtained in this way are shown as bar graphs in Figure 7, Figure 8, Figure 9, and Figure 10, respectively.

## (iii) Results

**[0079]** As seen in Figure 7, the cell growth effects on IGS in concentrations of 0.3, 1, 3, 10, 30, and 100  $\mu\text{M}$  were 98, 103, 106, 124, 134, and 133%, respectively, as compared with that on the control. Significant difference ( $p < 0.01$  in all the cases) could be noted when the concentration was 10  $\mu\text{M}$  or higher.

**[0080]** As seen in Figure 8, the cell growth effects on PIG in concentrations of 0.3, 1, 3, 10, 30, and 100  $\mu\text{M}$  were 101, 103, 113, 132, 131, and 134%, respectively, as compared with that on the control. Significant difference (3  $\mu\text{M}$ :  $p < 0.05$ ; 10, 30, and 100  $\mu\text{M}$ :  $p < 0.01$ ) could be noted when the concentration was 1  $\mu\text{M}$  or higher.

**[0081]** As seen in Figure 9, the cell growth effects on PIGS in concentrations of 0.3, 1, 3, 10, 30, and 100  $\mu\text{M}$  were 104, 103, 108, 110, 127, and 133%, respectively, as compared with that on the control. Significant difference (3 and 10  $\mu\text{M}$ :  $p < 0.05$ ; 30 and 100  $\mu\text{M}$ :  $p < 0.01$ ) could be noted when the concentration was 3  $\mu\text{M}$  or higher.

**[0082]** As seen in Figure 10, the cell growth effects on GPIG in concentrations of 0.3, 1, 3, 10, 30, and 100  $\mu\text{M}$  were 105, 108, 118, 133, 134, and 138%, respectively, as compared with that on the control. Significant difference (0.3  $\mu\text{M}$ :  $p < 0.05$ ; 1, 3, 10, 30, and 100  $\mu\text{M}$ :  $p < 0.01$ ) could be noted when the concentration was 0.3  $\mu\text{M}$  or higher.

**[0083]** The test was further conducted on epidermal cells, dermal fibroblasts and hair papilla cells to find that the epithelial cell growth promoter of the present invention exhibited the same cell growth-promoting effect on the epidermal cells as that on the HBKs along with no influence on the dermal fibroblasts and the hair papilla cells.

## Example 6

**[0084]** A 1mg portion of the pentapeptide obtained in Example 3 was dissolved in 300  $\mu\text{l}$  of water for injection (product code "057-00456", a product by Otsuka Pharmaceutical Co.), to which 200  $\mu\text{l}$  of propylene glycol (product code "161-05006", a product by Wako Pure Chemical Industries) and 500  $\mu\text{l}$  of ethyl alcohol (product code "057-00456", a product by Wako Pure Chemical Industries) were then added and they were mixed together to prepare an epithelial cell growth promoter in a concentration of 1 mg/ml.

**[0085]** Ten 7-week-old C3H/He female mice were raised for acclimatization for 1 week, and the hair on the dorsal of each mouse in the telogen phase of a hair cycle was shaved with an electric clipper and an electric shaver to prepare experimental animals.

**[0086]** Next, these experimental animals were divided into two groups each containing five mice. The epithelial cell growth promoter was applied at 100  $\mu$ l/mouse once a day to the shaved area of the first group from the 3rd day after shaving on, while only a mixture of water for injection, propylene glycol, and ethyl alcohol (3:2:5 by volume) was applied as a control to the second group. When pictures of the shaved areas were taken with a digital camera on the 14th day after application, evident hair growth promotion was observed in the group (B) with application of the epithelial cell growth promoter of the present invention, as compared with that observed in the control group (A).

**[0087]** Next, these images were computer-processed to calculate with image analysis software the ratio of the hair-regrowth area (the number of pixels of hair-regrowth area/the number of pixels of shaved area) as percentages. A significant difference test was performed by Student's t test (software "StatVIEW J-4.02", a product by Abacus Concepts). When a significance level was less than 5% ( $p < 0.05$ ), it was concluded that there was significant difference.

**[0088]** The results are shown in Figure 11. In this figure, white portions represent unhaired portions, and black portions represent haired portions. As seen from the results, the ratio of the hair-regrowth area of the control group (A) was  $36.9 \pm 5.7\%$ , whereas the ratio of the hair-regrowth area of the group (B) with application of the epithelial cell growth promoter of the present invention was  $66.6 \pm 3.5\%$ , to show that the epithelial cell growth promoter of the present invention promoted hair regeneration significantly ( $p < 0.01$ ).

#### Example 7

**[0089]** To confirm the cell selectivity of the oligopeptide obtained in Example 3 as an epithelial cell growth promoter, the presence or absence of the cell growth-promoting effect on cells existing in skin tissues, that is, HBKs, epidermal cells, dermal fibroblasts, and hair papilla cells, was

examined in the following manner. Incidentally, the same culture medium and test medium as in Example 5 were used.

(1) Preparation of HBKs, epidermal cells, dermal fibroblasts, and hair papilla cells

**[0090]** The skin of a 5 day-old C3H/HeN mouse was aseptically collected and cut with a surgical knife into samples of strips of approximately 2-mm-wide skin slices. The above-mentioned samples were immersed in a solution prepared by dissolving 500 U/ml of dispase (lot No. 0101, a product by GODO SHUSEI CO.) in DMEM (product code "D-5523", a product by Sigma) containing 5% by mass of FBS (lot No. 0101, a product by Cansera International Inc.), and then kept standing at 4°C for 16 hours. Subsequently, epidermis tissues were ablated with tweezers from the skin slices and separated from dermis tissues. The thus obtained epidermis tissues were subjected to the separation of epidermal cells, and the dermis tissues were subjected to the separation of HBKs and dermal fibroblasts.

**[0091]** The thus obtained dermis tissues were immersed in PBS (-) (product code "P-4417", a product by Sigma) and cut into strips with ophthalmological scissors. The strips were immersed into a solution prepared by dissolving 0.2% by mass of collagenase (lot No. 001014W, a product by Nitta Gelatin Inc.) in DMEM containing 5% by mass of FBS, and then digested at 37°C for 1 hour. The thus obtained dermis digestion solution was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and the residue was supplemented with PBS (-) and gently pipetted to prepare a dermis suspension.

**[0092]** For separating dermal fibroblasts and hair bulbs, this dermis suspension was kept standing for 15 minutes to precipitate only the hair bulbs. The "standing to precipitation" procedure was repeated three times. The thus obtained hair bulbs were immersed in a solution preparing by dissolving 0.25% by mass of trypsin in 2.65 mM aqueous EDTA solution, and then treated at 37°C for 5 minutes to prepare a dispersion solution of HBKs.

**[0093]** Next, this dispersion solution was centrifuged at 1000 rpm for 5 minutes. After this centrifugation, the supernatant was removed. The thus obtained HBKs were dispersed in a culture medium, then inoculated

into a collagen-coated 96-well microplate, and cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**[0094]** On the other hand, dermal fibroblasts floating in the supernatant by the first "standing to precipitation" procedure were collected and centrifuged at 1000 rpm for 5 minutes. After this centrifugation, the supernatant was removed. The thus obtained dermal fibroblasts were dispersed in a culture medium, then inoculated into a 96-well microplate, and cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**[0095]** The above-mentioned epidermis tissues were immersed in a solution prepared by dissolving 0.25% by mass of trypsin in 2.65 mM aqueous EDTA solution, and then treated at 37°C for 5 minutes to prepare a dispersion solution of epidermal cells.

**[0096]** Subsequently, this dispersion solution was centrifuged at 1000 rpm for 5 minutes, and the supernatant was removed. The thus obtained epidermal cells were dispersed in a culture medium, then inoculated into a collagen-coated 96-well microplate, and cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**[0097]** Moreover, human hair papilla cells (product code "602-05", a product by TOYOBO CO.) were dispersed in a culture medium, then inoculated into a collagen-coated 96-well microplate, and cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

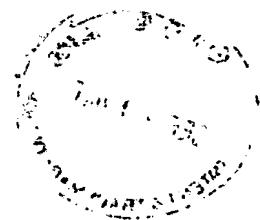
## (2) Cell growth test

### (i) Sample preparation

**[0098]** A 1mg portion of the pentapeptide obtained in Example 3 was added to 232.8 µl of a test medium and dissolved by stirring to prepare a 10 mM solution. Subsequently, this 10 mM solution was stepwise diluted to prepare 6 types of sample solutions each in a concentration of 100 µM (No. 1), 30 µM (No. 2), 10 µM (No. 3), 3 µM (No. 4), 1 µM (No. 5), and 0.3 µM (No. 6). Only a test medium was used as a control.

### (ii) HBK and epidermal cell growth tests

**[0099]** After 24 hours from the inoculation of the cells, the culture solutions were removed, and the cultured cells were washed with an MCDB153 solution. Each of the above-mentioned sample solutions was added to the cultured cell at 100 µl/well and cultured at 37°C in an atmosphere



control. Significant difference ( $p < 0.01$ ) could be noted when the concentration was 1  $\mu\text{M}$  or higher.

**[0104]** This demonstrated that the epithelial cell growth promoter of Example 3 dose-dependently exhibited the HBK growth-promoting effect in a concentration of 1  $\mu\text{M}$  or higher.

**[0105]** As seen in Figure 13, the effects of the sample solutions on the epidermal cells were 102% (No. 6), 110% (No. 5), 118% (No. 4), 124% (No. 3), 127% (No. 2), and 127% (No. 1), respectively, as compared with that of the control. Significant difference (1  $\mu\text{M}$ :  $p < 0.05$ , 3  $\mu\text{M}$  or higher:  $p < 0.01$ ) could be noted when the concentration was 1  $\mu\text{M}$  or higher.

**[0106]** This demonstrated that the epithelial cell growth promoter of Example 3 dose-dependently exhibited the growth-promoting effect on epidermal cells as well as HBKs in a concentration of 1  $\mu\text{M}$  or higher.

**[0107]** On the other hand, as seen in Figures 14 and 15, the epithelial cell growth promoter of Example 3 was shown to have no influence on dermal fibroblasts and hair papilla cells.

**[0108]** These facts demonstrated that the epithelial cell growth promoter of Example 3 exhibited selective cell growth-promoting effect on HBKs and epidermal cells classified into epithelial cells.

**[0109]** In this way, the epithelial cell growth promoter of the present invention exhibits selective cell growth-promoting effect on HBKs and epidermal cells classified into epithelial cells so as not adversely to affect cells other than epithelial cells constituting skin tissues, when used as a liniment such as, for example a lotion.

#### Industrial Applicability

**[0110]** The compound of the present invention can be employed as an epithelial cell growth promoter for hair growth and skin regeneration. According to the present invention, a novel excellent epithelial cell growth promoter having not only hair growth effect but also skin regeneration effect and therapeutic effect on atopic dermatitis is provided.